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THE ROLE OF OMPD IN ADHERENCE OF SALMONELLA ENTERICA SEROVAR  
TYPHIMURIUM TO MACROPHAGES AND THE EFFECTS OF  
MANNAN-BINDING LECTIN IN INITIAL ATTACHMENT

BY

Donna Louise Terrio

B.S., University of Massachusetts, 1994

THESIS

Submitted to the University of New Hampshire

in Partial Fulfillment of

the Requirements for the Degree of

Master of Science

In

Microbiology

September, 2008

UMI Number: 1459518

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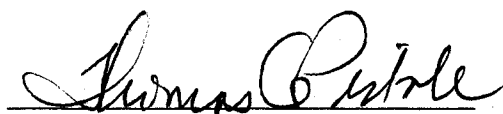
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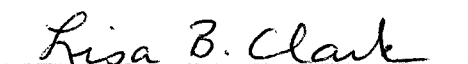
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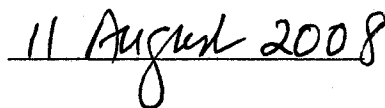
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Date

## **ACKNOWLEDGEMENTS**

I would like to thank Dr. Pistole for his support and guidance during my studies here at UNH. I would also like to thank Dr. Laudano for his expertise and advice during critical periods of my research. I am also deeply indebted to my fellow graduate students for all of their support, encouragement and last, but not least, their friendship.

## **DEDICATION**

***To my father and mother  
with love and gratitude***

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## ABSTRACT

### THE ROLE OF OMPD IN ADHERENCE OF SALMONELLA ENTERICA SEROVAR TYPHIMURIUM TO MACROPHAGES AND THE EFFECT OF MANNAN-BINDING LECTIN IN INITIAL ATTACHMENT

by

Donna Terrio

University of New Hampshire, September, 2008

The binding of macrophages to the outer membrane porin proteins, OmpC and OmpD, and the effects of mannan-binding lectin (MBL) were investigated. MBL was isolated from pig serum in a multi-step affinity chromatography process and the purity of the samples was determined via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. An Immunoglobulin G (IgG) Enzyme-Linked Immunosorbent Assay (ELISA) was used to detect MBL. Porcine MBL was present in serum in approximately 30, 32 and 34 kDa monomeric forms. Bacterial binding assays were performed using *Salmonella enterica* serovar Typhimurium wild-type, *ompC*- deficient and *ompD*-deficient bacterial strains. These experiments were repeated in the presence of increasing concentrations of either porcine or human MBL.

Adherence of U937 cells to both the wt and *ompC*- deficient strains increased in a dose dependent manner. In contrast adherence of U937 cells to the *ompD*- deficient strain did not change significantly in the presence of either porcine or human MBL. Inhibition assays using an anti-MBL monoclonal antibody supported these results. We conclude that the outer membrane protein D mediates the attachment of U937 cells and that MBL influences *OmpD* adherence to this macrophage cell line.

## INTRODUCTION

*Salmonella* infections continue to pose a serious threat to public health and agriculture. During recent years there has been a sharp increase in bacterial resistance to antibiotics making it ever more difficult to treat and prevent infection. In the United States alone, nontyphoidal *Salmonella* is responsible for approximately 1.4 million foodborne infections each year and accounts for more deaths than do infection with any other foodborne bacterial pathogen (16). In addition, the increase in *Salmonella* infections has had an economic impact. In the human population, the estimated annual costs related with *Salmonella* infections are \$0.5-\$3.2 billion/year (15).

There are over 2500 known *Salmonella enterica* serovars, producing distinct disease symptoms in different animals. *Salmonella enterica* serovar Typhimurium, a Gram-negative facultative anaerobe, produces gastroenteritis in humans and typhoid-like disease in mice (1). On the other hand, *Salmonella enterica* serovar Typhi grows exclusively in humans, causing typhoid fever (17). *Salmonella* is transmitted primarily via the oral route by ingestion of contaminated water or animal products. The infective dose for humans is approximately  $10^6$  -  $10^8$  colony forming

units (CFU), although in many cases it has been shown to be substantially lower (39). The onset of disease can be anywhere between 5-20 days. The variance is due to several factors, including the number of bacteria ingested and the health of the individual (40).

Orally ingested *Salmonella* penetrate the intestinal mucosa migrating through specialized M cells (2), where they interact with macrophages. During bacterial infection, macrophages serve as professional phagocytes and key effectors of the innate and adaptive immune responses (14). Although macrophages are important host defense cells, *Salmonella* Typhimurium has been shown to benefit from the macrophage's phagocytic actions. *Salmonella* is able to reside intracellularly, where it can replicate inside specialized vacuoles within macrophages (3). Studies have shown that the growth rate of the bacteria within these vacuoles changes over time and is affected by the rapid acidification of the vacuole (40, 41). If acidification is prevented then the bacteria cannot multiply, although they are able to survive (41). Therefore, the antimicrobial acidification process allows time for the bacteria to propagate and greatly affects the outcome of disease.

Macrophages have developed specific receptors that recognize

certain conserved motifs on pathogens. For example, the mannose receptor recognizes and preferentially binds to carbohydrates containing either mannose or fucose (25). It has been proposed that this carbohydrate-specific receptor is part of a primitive immune recognition system (5). There are other macrophage receptors, such as the scavenger receptors, that have also been found to bind mannose (8,9). However, for most of these molecules, the nature of the receptor and the signaling pathways they use remain undefined.

Mannan-binding lectin (MBL), a C-type lectin, also recognizes and binds to sugars on microbial cell surfaces. Binding of MBL and its associated serine proteases, MASP-1 and MASP-2, activates the lectin complement pathway (4,5). There are two additional MASPs, MASP-3 and MASP-4, but very little is presently known about their function (4,9). These enzymes are able to cleave C4 and C2 to form C4bC2a, a C3 convertase that enzymatically splits numerous molecules of C3 into C3a and C3b (5,6,9). Small fragments produced during the cleavage of the complement proteins act as chemokines and recruit phagocytes to the area. Finally, the terminal components of complement bind together and attach to the bacterial membranes, resulting in the formation of pores and the lysis of the bacteria (8). MBL may also interact directly with



phagocytic cells to promote the opsonization of bacteria. That is, it may influence phagocytosis in the absence of complement activation through an interaction with one or more collectin receptors (3,6,8). The latter relationship was the focus of the current study.

The immune response to bacterial infection is initiated by the ability of the host to recognize pathogen-associated molecular patterns (PAMPs) on bacterial cell surfaces (7,28,36). Host cells discriminate between self and non-self through families of bacterial binding receptors that are designed to recognize a few highly conserved structures present in many different microorganisms (36,40). These receptors are called pattern recognition receptors (PRRs). PRRs become activated in response to binding specifically to PAMPs (4,25,26). Because different pathogens express their own array of PAMPs, the host has evolved multiple PRRs to recognize specific microbial motifs (3,4,26,28,36). The repeating sugar units located on the cell surface of *Salmonella* Typhimurium form a specific three dimensional pattern (7,28). It is this defined motif that mannan-binding lectin recognizes (4,9,26).

While *Salmonella* has been shown to express a large array of virulence factors, evidence suggests that microbial outer membrane porin proteins

are key surface associated molecular complexes involved in initial recognition of pathogens by host cells (11,12). Porins exist as either homo- or heterodimers and are involved in transport of small hydrophilic compounds into and out of the cell (7,10,11). These proteins play an essential role in the survival of the cell, and their study is of great interest with respect to potential medical applications. Porins will be discussed in greater detail later in this report.

The overall goal of this project was to help elucidate the "cross-talk" involved during the early infection process between *Salmonella* Typhimurium and host cells. The following objectives were designed to meet this goal. The first objective was to determine whether MBL enhances the adherence of human macrophages to *Salmonella*. Previously in our lab, porcine serum had been partially purified in an attempt to isolate MBL. However, a more purified sample was needed to continue research on this project. Therefore, the goal here was to develop an assay that would separate MBL from other proteins in the serum. Then a series of binding assays were performed using the purified porcine MBL, human MBL, and *Salmonella*. The goal was to determine whether MBL enhances macrophage attachment, engulfment and internalization of the bacteria.

The second objective was to determine whether outer membrane proteins – porins – are involved in macrophage recognition by salmonellae. Comparative studies were performed using a wildtype parent strain and two different mutants deficient in a specific porin: 1) an *ompC*- deficient strain (*ompC*::Tn10); and, 2) an *ompD*- deficient strain (*ompD*::Tn5). Microbial attachment in both studies was determined visually by light microscopy.

## **CHAPTER ONE**

## CHAPTER 1

### Isolation of Mannan-Binding Lectin from Porcine Serum

#### ABSTRACT

Author: Terrio, Donna

Type of Publication: Unpublished

Mannan-binding lectin (MBL) is a C-type lectin that recognizes and binds to carbohydrates on bacterial cell surfaces. Human MBL has been characterized and extensively studied, however, very little is known about porcine MBL. In this study, pig MBL was isolated by a multi-step affinity chromatography process and the purity of the samples was determined via SDS-PAGE under reducing conditions and an Immunoglobulin G (IgG) Enzyme-Linked Immunosorbent Assay (ELISA). It was found that porcine MBL is present in serum in forms with a reduced molecular mass of approximately 30, 32 and 34 kDa.

## INTRODUCTION

The shortage of human donor organs and tissues for transplantation has led researchers to consider xenotransplantation as a therapeutic option. Large advances have been made in the use of certain pig tissues in human transplants. However, rejection of these tissues has remained an obstacle. Pig organs have certain epitopes which are recognized by specific human antibodies. This can induce a hyperacute rejection by triggering the complement cascade, which involves the protein mannan-binding lectin (MBL). Presently very little is known about the function of porcine mannan-binding lectin (pMBL). The study of pMBL may aid in the development of strategies to circumvent xenograft rejection.

The primary structure of human MBL (hMBL) is a 96 kDa helical molecule (23,24). It is comprised of three identical 32 kDa chains. Each monomer consists of a "cysteine-rich" N-terminal region, followed by a collagenous region, a "neck" region, and finally a C-terminal carbohydrate-recognition domain (CRD), where the binding of sugars on microbial cell surfaces occurs (9,18,21). In addition, studies have shown that during

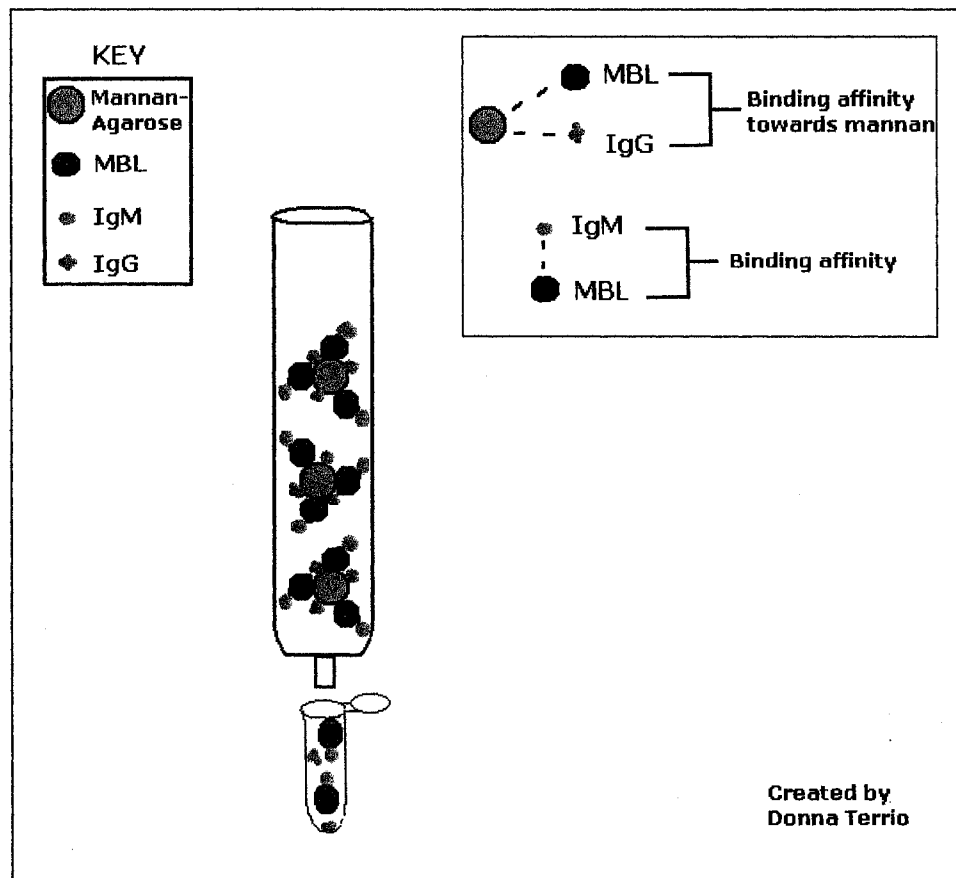
sodium didecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), under reducing conditions, pMBL separates into three bands with approximate molecular masses of 30, 32, and 34 kDa (20). In contrast, similar experiments have shown that hMBL reduces to a single 32 kDa band (21).

Previous studies in our laboratory have shown that pMBL mediates attachment of *Salmonella* Typhimurium to human U937 macrophage cells (I. Daniels, thesis). However, the MBL was incompletely purified, i.e., the final sample was contaminated with IgG. Since IgG is able to enhance the binding of bacteria to macrophages, it was not known whether MBL, IgG, or both mediated the attachment. Therefore, prior to the onset of the binding assays discussed later in chapter 2, a method was developed to further purify the MBL.

In this study, pMBL was isolated by a multi-step affinity chromatography process and the purity of the samples was determined via SDS-PAGE under reducing conditions and an Immunoglobulin G (IgG) Enzyme-Linked Immunosorbent Assay (ELISA). A mannan-agarose column was used during Phase 1 of the purification process. The eluted Phase 1 fractions contained MBL as well as IgG (fig. 1.1). To extract these

immunoglobulins from the sample a protein A-agarose column was used during Phase 2 of the purification process. Protein A is a protein found on the surface of *Staphylococcus aureus* strains that binds specific subsets of immunoglobulins. It has been proposed that binding of host immunoglobulins helps camouflage the bacterium from the immune system.





**Fig. 1.1. Schematic representation of the binding of MBL, IgG and IgM during affinity purification on mannan-agarose.** MBL and IgG both have an affinity toward mannan and thus bind to the mannan-agarose beads in the column in Phase 1 of the purification process. In addition, IgM has an affinity towards MBL. Therefore, the Phase1 fractions contained MBL as well as IgG and IgM.

## MATERIALS AND METHODS

**Chemicals.** All chemicals used were obtained from Sigma-Aldrich Company (St. Louis, MO), except where otherwise noted.

**Porcine serum stock.** Pig serum was generously donated by Dr. D. Bobilya (UNH, Durham, NH).

**Chromatography Supplies.** All equipment used in the purification process was obtained from Bio-Rad (Hercules, CA), unless otherwise noted.

**Anti-IgG antibody.** An anti-porcine IgG antibody ELISA was used (Alpha Diagnostic International, Inc., San Antonio, TX).

**Isolation of MBL from pig serum. Phase 1.** The purification process was carried out at 4 °C, except where otherwise stated. A pool of porcine serum, after addition of polyethylene glycol (PEG) 3350 to 10% (w/v), was stirred for 2 h. Precipitated proteins were pelleted by centrifugation for 10 min. at 8000 *g*. The pellets were dissolved in TBS-TCa<sup>2+</sup> buffer (50 mM Tris/1M NaCl/20 mM CaCl<sub>2</sub>/0.05% Tween 20, pH 7.8), and insoluble

materials were removed by centrifugation for 5 min at 8000 **g**. The supernatant was mixed for 2 h with 10 ml of mannan-agarose that was previously equilibrated with TBS-TCa<sup>2+</sup>. The resin was recovered by centrifugation for 5 min. at 1000 **g** and packed into a column after washing with TBS-TCa<sup>2+</sup>. The column was further washed with 200 ml of TBS-TCa<sup>2+</sup> and then eluted with TBS-TEDTA (TBS-TCa<sup>2+</sup> where 20 mM CaCl<sub>2</sub> was replaced with 10 mM EDTA). The EDTA eluate was made 40 mM with respect to CaCl<sub>2</sub> and reapplied to the column. After washing the column with TBS-TCa<sup>2+</sup>, bound proteins were eluted with 100 mM mannose in TBS-TCa<sup>2+</sup>. MBL-containing fractions, as judged by SDS-PAGE, were pooled. A separate aliquot was saved for later analysis (to be compared to the final purified MBL).

**ISOLATION OF MBL. Phase 2.** The pooled MBL- containing sample was first applied to a desalting column since high salt concentrations may increase non-specific binding. To elute the proteins, binding buffer (80% glycine, 20% sodium chloride, pH 9.0) was added. The eluate was collected and applied to a protein-A agarose column. The protein A column consists of purified protein A coupled to crosslinked agarose beads. The protein A column was designed to purify IgG. However, in our research we used the protein-A agarose beads to bind the IgG that

remained in the crude MBL sample (Phase1), while allowing the MBL to pass through, hence, purifying the MBL. The fractions containing the MBL were saved for further analysis.

After washing the column with binding buffer (80% glycine, 20% sodium chloride, pH 9.0) two times, any IgG that had adhered to the beads was removed with an elution buffer (65% citric acid, 35% trisodium salt dehydrate, pH 3.0). The eluate was collected and saved for further analysis. The collected samples were concentrated in an Apollo20 concentrator and washed with TBS-TCa<sup>2+</sup>, pH 7.8, at 1200 **g**, for 15 min. This step was repeated three times.

**BRADFORD-LIKE ASSAY.** To determine protein concentration in the samples a Bio-Rad protein assay was performed according to the protocol provided by the company. Briefly, Bio-Rad protein dye reagent was diluted and filtered just prior to use. Bovine Serum Albumin (BSA) standards were prepared containing a range of 20 to 400 micrograms of protein in 100 µl volumes, in separate 15 ml tubes. Diluted dye reagent (5 ml) was added to each tube and incubated for approximately 5 minutes and absorbance was read at 590 nm. To create a standard curve the absorption readings were plotted on a graph and an R-value was generated by which the unknown samples were analyzed.

## **SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS**

**(SDS-PAGE).** SDS-PAGE was carried out using a phosphate buffer system as described by Laemmli (45). The total polyacrylamide concentration was 12% for the separating gel. One volume from each purified sample was added to one volume of sample buffer (1:1). To examine proteins under reducing conditions, samples were heated for 5 minutes at 90°C in the presence of 50 mM dithiothreitol. A low molecular weight (LMW) standard marker from Bio-Rad was used. SDS-PAGE was performed at a constant voltage of 200. Silver stain was used to visualize the protein bands.

The protein concentrations that were determined in the Bio-Rad protein assay were based on the total protein in each sample. Since the Phase 1 sample should have contained both MBL and IgG it was not known how much of the total protein was MBL and how much was IgG. Therefore, to find comparative MBL and IgG concentrations a series of gels were run using varying protein concentrations from the Phase 1 and Phase 2 samples. Gels were then run using the established protein concentrations.

Approximately 25 µg total protein of the Ph1 sample was applied to these latter gels and approximately 15 µg total protein of the Phase 2 samples was applied. Lastly, to further determine the purity of the Phase 2

MBL sample, decreasing protein concentrations were loaded into one gel.

**SILVER STAINING SDS-PAGE GELS.** For visualization purposes, gels were silver stained per the protocol supplied by the company (Bio-Rad). Briefly, all steps were completed at room temperature and were under constant shaking conditions. Working solutions were prepared no longer than 24 hours prior to use. Gels were first fixed for 30 minutes in a fixing solution, then removed and placed in a sensitizing solution and incubated for 30 minutes. Gels were washed three times for 5 minutes in dH<sub>2</sub>O, then placed in silver staining solution and incubated for 20 minutes. The gels were washed twice for 1 minute, and placed in a developing solution until bands appeared (~1-5 minutes). Finally, the reaction was stopped by placing gels in a stopping solution for 10 minutes and washed three times for 5 minutes, then sealed in Kodak photographic clear plastic film.

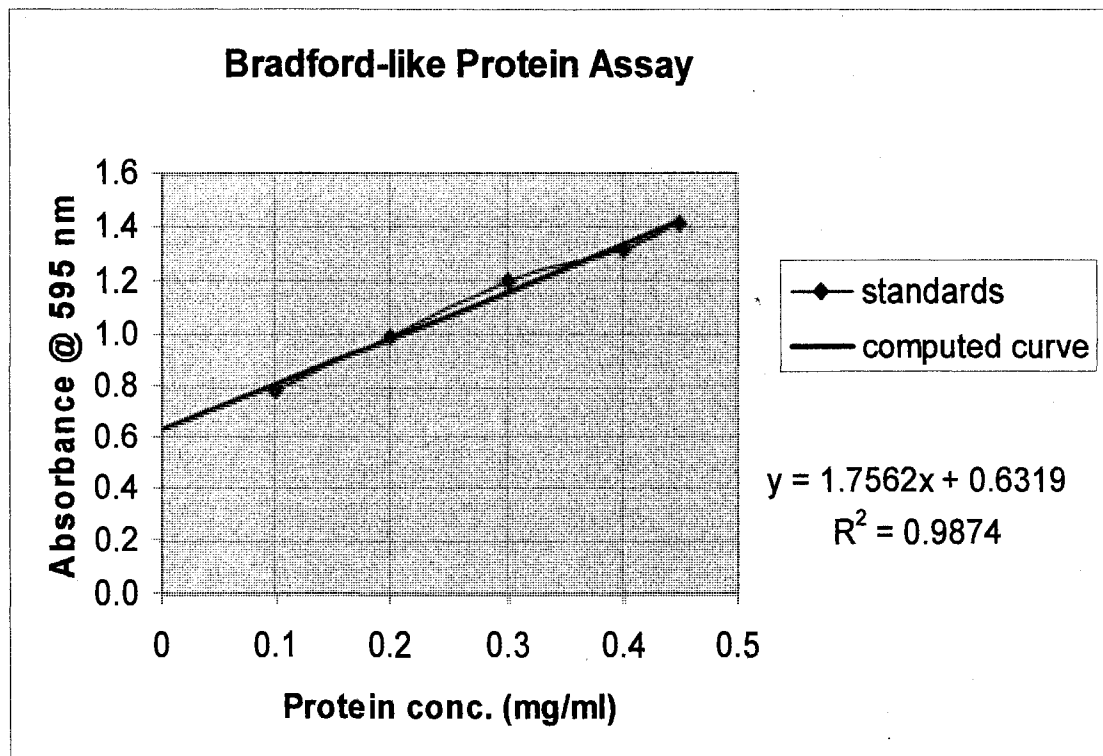
**IMMUNOGLOBULIN G (IgG) ENZYME-LINKED IMMUNOSORBENT ASSAY(ELISA).** 96-well plates pre-coated with anti-porcine IgG antibody were incubated with varying concentrations of IgG at 37°C. Plates were washed three times with PBS buffer containing 0.5% Tween-20. IgG was detected by addition of anti-pig IgG-horseradish peroxidase conjugate to

each well and incubated at 20°C for 1 hr. The plates were washed four times and 50 µl of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic) acid (ABTS) was added to each well and the plates were read at 405 nm using a microplate reader. All ELISA experiments were performed in triplicate and the mean average was calculated for each experimental condition.

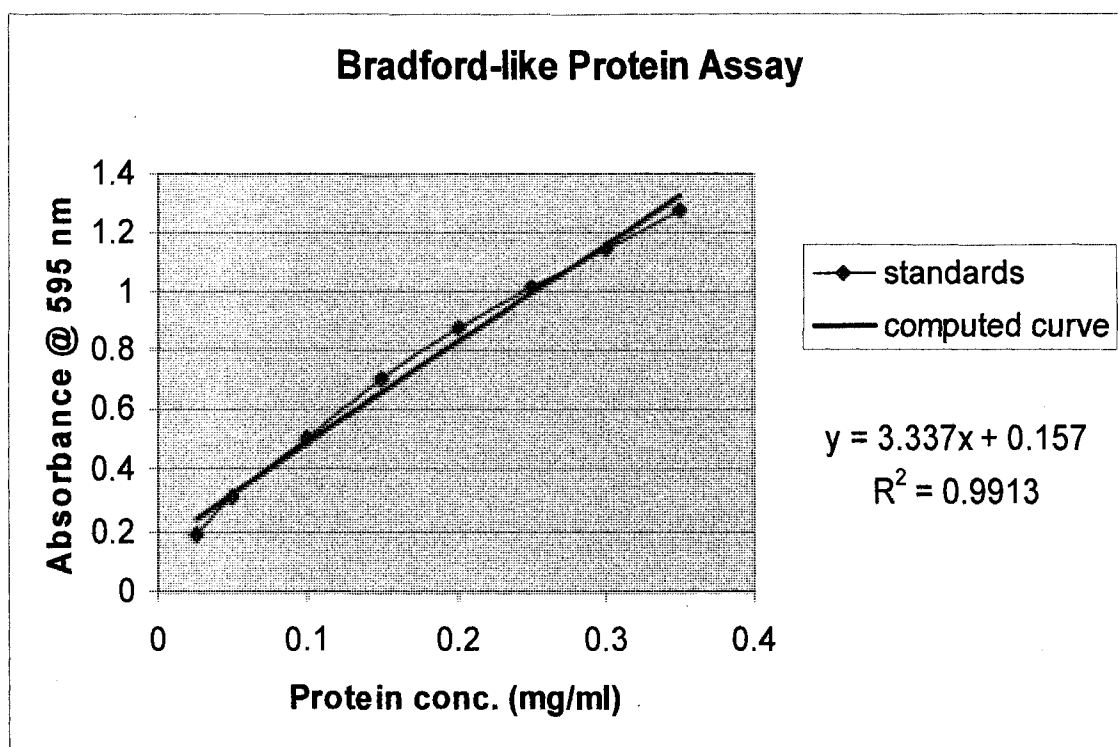
## RESULTS

**Determining protein concentration of the potential MBL-containing fractions.** Two separate Bio-Rad protein assays were performed to determine total protein concentration of the phase one and phase two samples. The first protein assay analyzed the Phase 1 sample and the second assay analyzed the two separate Phase 2 samples. The samples were measured on a spectrophotometer and absorption was read at 595 nm. The BSA standards were plotted and a y-formula was generated, whereby protein concentration was calculated. The  $R^2$  was the fraction of the total squared error. The  $R^2$  value was above 0.95 in both experiments, indicating a good model. The data were collected and the protein concentration was compared between the two samples. The Phase 1 fraction contained ~177  $\mu\text{g}$  of protein/ml (Fig. 1.2). The Phase 2 sample contained approximately 23.6  $\mu\text{g}$  of protein/ml (Fig. 1.3).





**Fig. 1.2. Protein concentration determination of the Phase 1 MBL sample.** The absorption values of the BSA standards were measured at 595 nm and plotted. The protein concentration of the samples were determined by using the generated formula. The  $R^2$  value above was approximately 0.99. Absorbance was read on three samples. The average absorbance reading was 0.943. Protein concentration for the Phase 1 sample = 177  $\mu\text{g/ml}$ .



**Fig. 1.3. Protein concentration determination of the Phase 2 MBL sample.** Absorption of the BSA standards was measured at 595 nm and plotted on a graph. The protein concentration of the MBL Phase 2 sample was determined by using the generated formula as done in Fig. 1.2. Absorbance was read on three samples and the average absorbance value was 0.236. The protein concentration for the MBL Phase 2 sample = 23.6  $\mu\text{g/ml}$ .

**Determining purity of the MBL-containing samples via SDS-PAGE.** The purity of each chromatographic fraction was analyzed by SDS-PAGE under reduced conditions (fig. 1.4 and 1.5). On figure 1.4, lane 1 contained the low molecular weight marker proteins. Lane 2, contained pooled fractions from the first chromatography step (Phase 1). Lane 3 corresponds to the pooled MBL-containing fractions obtained from Phase 2. Lane 4 contains the IgG from the elution step in Phase 2.

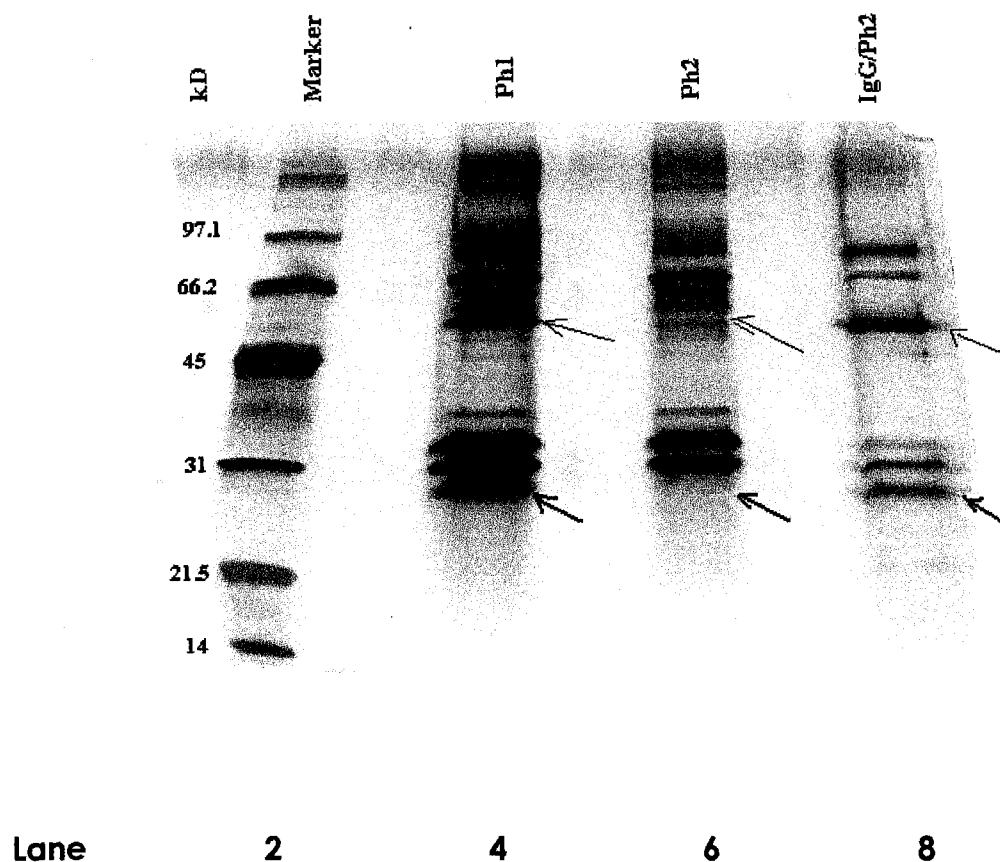
On figure 1.4 the distinct polypeptide bands show a molecular weight of about 50-kDa which corresponds to pig IgG heavy chains. Also, the band at molecular weight of approximately 25 kDa corresponds to pig IgG light chains. There are bands at approximate molecular weights of 30, 32, and 34 kDa corresponding to the three monomeric forms of pig MBL.

The series of arrows across the top portion of the gel on fig. 1.4 points to the 50 kDa location. Lane 2 shows a band at this location indicating the presence of IgG heavy chain. Lane 3 shows the deletion of that band in the Phase 2 sample, indicating that the IgG was extracted from the Phase 1. Lane 4 shows the presence of the 50 kDa band in the Phase 2-IgG sample.

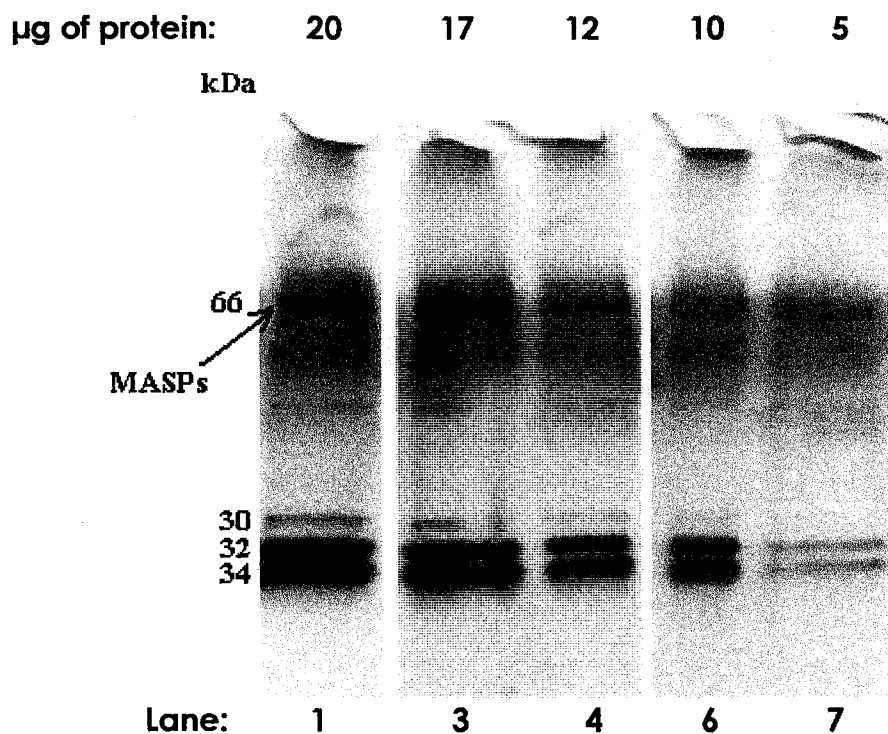
The series of arrows across the bottom portion of the gel on fig. 1.4 points to the 25 kDa location. Lane 2 shows a band at this location

indicating the presence of IgG light chain, in the partially purified MBL sample (Phase 1). Lane 3 shows the deletion of this band in the more purified MBL sample from Phase 2. Lane 4 shows the presence of IgG light chain in the fractions eluted from the IgG column at the end of the Phase 2 process.

To further determine the purity of the Phase 2 MBL sample, decreasing protein concentrations were loaded into one gel (fig. 1.5).



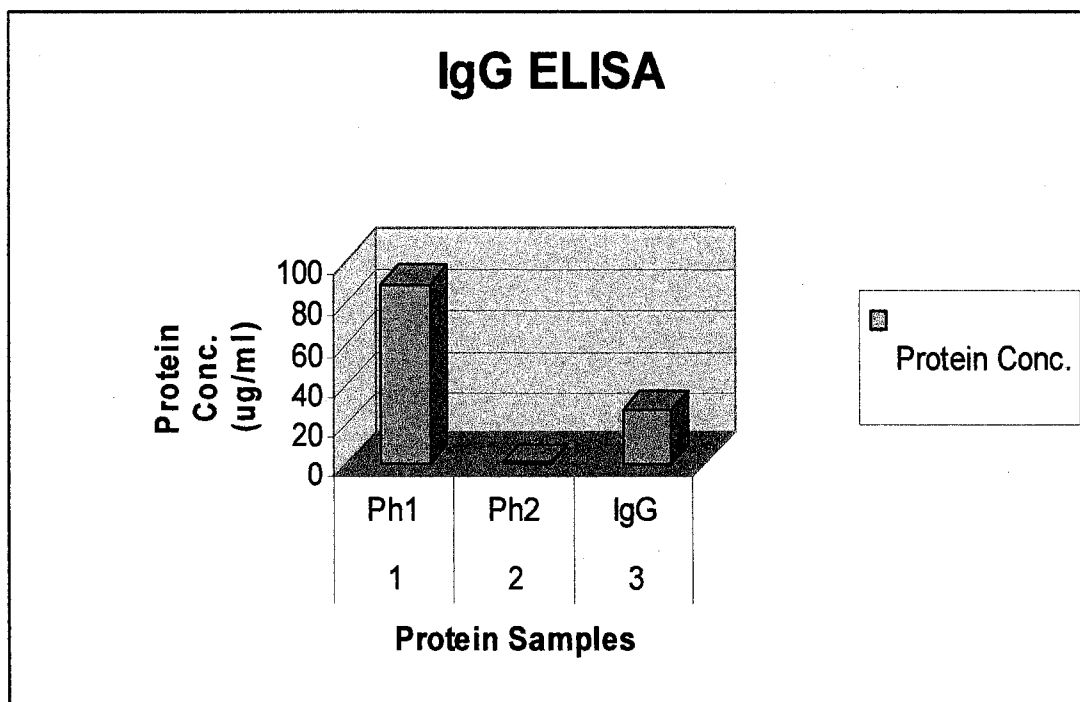
**Fig. 1.4. SDS-PAGE gel analysis of the Phase 1 and Phase 2 samples.** The arrows across the top designate the 50 kDa IgG band. The bottom arrows designate the 25 kDa IgG light chain band. Lane 6 (Phase 2 MBL-containing sample) shows the disappearance of the 50 kDa IgG heavy chain and the 25 kDa IgG light chain. Both chains are present in lane 8 (the IgG-containing sample captured during Phase 2). The bands in each well at ~ 66 kDa are most likely MBL associated serine proteases (MASPs). Proteins were separated with 12% polyacrylamide gel and visualized by silver staining.



**Fig. 1.5. SDS-PAGE (2-mercaptoethanol reduced) analysis of purified Phase 2 porcine MBL and associated serine proteases (MASPs).** The lanes contained decreasing concentrations of protein ranging from 20 µg to 5 µg of total protein. The arrow indicates the ~ 66 kDa MASPs. The bottom 3 bands are the MBL bands at ~ 30, 32, and 34 kDa. Proteins were separated with 12% polyacrylamide gel and visualized by silver staining. Lanes 2 and 5 remained empty. All lanes are from the same gel.

### **Determining the presence of IgG in the phase 2 fractions via**

**anti-IgG antibody ELISA.** To verify that IgG was extracted from the MBL sample during Phase 2 of the purification process an ELISA was performed. Protein samples were added to wells pre-coated with anti-pig IgG antibody. The anti-porcine polyclonal antibody recognized porcine IgG in the samples. The Phase 1 sample showed the highest concentration of IgG present at 88.4 µg/ml. The Phase 2 MBL-containing sample showed a sharp decrease in IgG concentration at 0.24 µg/ml. The IgG-containing fraction collected during Phase 2 showed a significant concentration of IgG, at 27.43 µg/ml. These results support the SDS-PAGE data, and verify that IgG was extracted from the MBL-containing Phase 2 sample.



**Fig. 1.6. IgG ELISA analysis of protein samples collected during Phase 1 and Phase 2 of the purification process.** Protein samples were added to wells pre-coated with anti-porcine IgG antibody. The Phase 1 sample showed the highest concentration of IgG at 88.4  $\mu\text{g/ml}$  (#1). The Phase 2 MBL-containing sample (#2) showed a sharp decrease in IgG concentration at 0.24  $\mu\text{g/ml}$ . The IgG-containing fraction collected during Phase 2 (#3), showed a significant concentration of IgG at 27  $\mu\text{g/ml}$ , verifying that IgG was extracted from the Phase 1 sample.



## DISCUSSION

Previously in our lab, porcine MBL was partially purified by a 2-step chromatography process (I. Daniels, thesis). In the current study this portion of the purification process was further developed and designated Phase 1. To isolate MBL a subsequent assay was developed and designated Phase 2. A column packed with mannan-agarose was used to purify MBL during Phase 1. Electrophoresis gel analysis showed impurities at this stage. Due to its affinity to mannan IgG was thought to be a possible contaminant. Therefore, the goal was to develop an assay that would remove the possible IgG contaminant, resulting in a more purified MBL sample. Since protein A has an affinity towards porcine IgG a Bio-Rad protein-A column was used to remove the IgG from the Phase 1 sample.

SDS-PAGE analysis revealed the presence of three monomeric forms of porcine MBL with approximate molecular masses of 30, 32 and 34 kDa. These data support previously published observations by Agah et al. (20). In contrast, human MBL has been characterized and shown to exist in a single isoform (4,23,24) with an approximate molecular mass of 32 kDa. Interestingly, Agah and colleagues also suggested that the three porcine

monomeric isoforms isolated by their methods are a single protein with post-translational modifications.

Electrophoresis results revealed protein bands of approximately 50 kDa that correspond to porcine IgG heavy chains and the 25 kDa protein bands that correspond to porcine IgG light chains. These bands were present in the partially purified MBL sample in Phase 1 and were virtually undetectable in the more purified MBL Phase 2 sample. In addition, the IgG that was removed from the partially purified MBL sample during Phase 2 of the purification process showed bands at both the 50- and 25- kDa location in lane 4. These data support the hypothesis that IgG was present in the Phase 1 sample and was successfully separated from the MBL sample during the protein-A chromatography portion of the purification process. Evidence that the IgG was captured was supported by anti-porcine IgG ELISA analysis. Results showed that there was a high degree of IgG present in the Phase 1 sample and the Phase 2-IgG sample. In addition, IgG was only present at very low levels in the Phase 2 MBL-containing sample, once again indicating that IgG was extracted from the Phase 2 sample. Our findings verified that a protein A column is an appropriate and useful step in the purification of MBL from porcine serum.

## **CHAPTER TWO**

## CHAPTER 2

### The Role of OmpD in the Adherence of U937 Cells to *Salmonella enterica* serovar Typhimurium in the Presence of Mannan-Binding Lectin

#### ABSTRACT

Author: Terrio, Donna

Type of Publication: Unpublished

The outer membrane porin protein D (OmpD) of *Salmonella enterica* serovar Typhimurium has been found to be a ligand for the human macrophage cell line, U937. For comparison purposes, bacterial binding assays were performed using *Salmonella enterica* Serovar Typhimurium wild-type and OmpC- and OmpD-deficient bacterial strains. These experiments were repeated in the presence of either porcine MBL (pMBL) or human MBL (hMBL). It was observed that binding of the U937 cells to wt and the *ompC* mutant strain increased when organisms were preopsonized with hMBL or pMBL. Ten-fold increases in human or porcine MBL concentrations induced increased binding activity in a dose-responsive manner in the wt strain, but had little effect on the *ompD* mutant strain. Inhibition assays using an anti-MBL monoclonal antibody supported these results. We conclude that OmpD mediates the

attachment to U937 cells and that both human and porcine MBL facilitates adherence.

## INTRODUCTION

Evidence suggests that outer membrane porin proteins are key surface-associated molecular complexes involved in initial recognition of pathogens by host cells (3,10,18,22,35). Understanding the mechanisms by which *Salmonella* stimulates host cellular responses in the initial stages of infection will aid us in the development of novel therapeutic drugs. In addition, studies in this area may lead to a deeper understanding of the biological bases of persistent infection commonly observed with a variety of *Salmonella* serotypes. The studies outlined here were designed to test the following hypotheses: 1) Mannan-binding lectin (MBL) enhances the binding of macrophages to *Salmonella* Typhimurium; and, 2) The outer membrane protein OmpD plays a role in the adherence of macrophages to *S. typhimurium* in the presence of MBL.

*Salmonella* express many different outer membrane proteins, including OmpC (~36 kDa), OmpF (~35 kDa) and in some serovars such as *Salmonella* Typhimurium, OmpD (~34 kDa) (7). The relative amounts of

OmpC, OmpF, and OmpD changes in response to various environmental conditions. For example, under anaerobic conditions OmpD expression is up-regulated, while OmpC is down-regulated (47).

Previous studies in our lab have shown that OmpC in *Salmonella* Typhimurium is a ligand for macrophage recognition in the mouse model (31). However, it was unknown whether OmpC is a ligand in certain human macrophage cell lines. Further studies in our lab showed that OmpD but not OmpC was a ligand for macrophage recognition using U937 cells – a human macrophage cell line (Hara-Kaonga and Pistole, 2004). The present project extended these studies to include the effects of human or porcine mannan-binding lectin on this system.

MBL binds to mannose O-polysaccharides found on the cell surface of *Salmonella* via a carbohydrate recognition domain (CRD). The spacing and orientation of the CRD defines what ligands the protein can target (8,26). MBL binds to *Salmonella* by means of a pattern-recognition mode of detection (4,25). Thus, MBL recognizes and binds to the specific three dimensional pattern formed by the repeating oligosaccharide units, thereby initiating contact with *Salmonella*. In vitro, MBL seems to recognize purified components of micro-organisms; however, the relationship between binding to these isolated

components does not always correlate with binding to the whole organism (1). It is possible that these components are released to block attachment of MBL to the whole organism (18). In addition, MBL does not always bind to organisms that contain mannose (2,9). These data suggest that there are other key factors involved in adherence. The majority of our knowledge is through empirical evidence, with a number of publications identifying organisms that bind MBL and a few studies that have explored how changes in the microbial surface alter MBL binding (4,7,9). We cannot currently predict the microbial targets of MBL binding, other than testing organisms with very high mannose (or *N*-acetyl-D-glucosamine or similar) glycans on their surface. For this reason, *Salmonella* Typhimurium is a model organism, since it is rich in cell-surface mannose carbohydrates.

## MATERIALS AND METHODS

**Chemicals.** All chemicals used were obtained from EM Science, Gibbstown, NJ, except where otherwise noted.

**Abbreviations.** Phosphate-buffered saline (PBS), Hanks Balanced Salt solution (HBSS), Luria-Bertani (LB) broth and agar, Fetal bovine serum (FBS), phorbol 12-myristate 13-acetate (PMA).

**Bacterial Strains and Growth Conditions.** *Salmonella* Typhimurium strain 14028 (ATCC) was used. The OmpC mutant (BHKC1) and OmpD mutant (BHKD1) were created by transposon mutagenesis in the 14028 parent strain (Hara-Kaonga and Pistole, 2004) (see table 2.1). The 3 strains were each grown anaerobically, under non-agitating conditions, in 50 ml of LB broth for ~8 h at 37°C and diluted 1: 20 in LB broth. The diluted culture was incubated anaerobically, without shaking, for 14-16 h at 37°C. The bacteria in the logarithmic phase were harvested by centrifugation at 9,000 x *g* at 4°C for 10 minutes, washed three times with PBS and resuspended in 5 ml of HBSS. Bacterial concentrations were measured using a spectrophotometer at 600 nm. These concentrations were confirmed by serial plating on LB agar. Colonies were counted visually.



Table 2.1. Bacterial Strains used.

Strain of <i>Salmonella enterica</i> serovar Typhimurium	Genotype	Source
14028	smooth wild-type	ATCC <sup>a</sup>
BHKC1	<i>ompC::Tn10</i>	Hara-Kaonga and Pistole. (29)
BHKC2	<i>ompD::Tn5</i>	Hara-Kaonga and Pistole. (29)

<sup>a</sup> American Type Culture collection

**Macrophages.** U937 cells obtained from ATCC were cultured in RPMI 1640 medium (ATCC) supplemented with 10% FBS at 37°C, 5% CO<sub>2</sub>. Cell viability was determined by trypan blue exclusion. U937 cells,  $\sim 5.0 \times 10^7$  cells per T75 flask, were treated with PMA (10 ng/ml) to promote differentiation and adherence and incubated overnight at 37°C, 5% CO<sub>2</sub>.

**Human Mannan-binding Lectin (hMBL).** hMBL was obtained from US Biologics, Swampscott, MA.

**Anti- porcine mannan-binding lectin antibody.** Anti- porcine MBL monoclonal antibody was obtained from Chemicon International, Inc., Temecula, CA.

**Binding Assays:** The PMA-treated U937 cells were centrifuged at 800 x **g** for 10 min. and the pellet was resuspended in fresh medium (RPMI 1640, 10% FBS) without PMA, then added to a series of 8-well chambered slides (Nunc, Kamstrup, Denmark).

After a 2 hour incubation at 37°C, 5% CO<sub>2</sub>, the U937 cells were washed with PBS, then separately incubated with the three *S. Typhimurium* strains (wild-type, *ompC* mutant, *ompD* mutant) at the following

macrophage/bacteria ratios: 1:10, 1:100, 1:500. This was done as a preliminary study to determine an optimum bacterial/macrophage ratio. Subsequent experiments were performed using 1:10 ratio only.

The assay was then repeated as above, with one additional step. Prior to adding the bacteria to the U937 cells in the chambered slides, the bacteria were first mixed with either porcine MBL or human MBL at increasing concentrations then incubated for 1 hour at 4°C. The MBL concentrations were as follows: 0.03 µg/ml, 0.30 µg/ml, and 3.0 µg/ml. After washing the chambered slides containing the U937 cells with HBSS, the varying bacteria/MBL concentrations were added to the wells (see table 2.2) and incubated for 2 hours at 37°C. Slides were fixed and stained and the mean number of attached bacteria per macrophage was determined by counting the number of adherent cells by light microscopy.

**Table 2.2. Contents of the various wells in the dose response binding assays.** The gray shaded areas were the controls.

Well #	U937	wt 14028	OmpC mutant	OmpD mutant	Porcine MBL ( $\mu\text{g/ml}$ )	Human MBL ( $\mu\text{g/ml}$ )
1	+					
2	+	+				
3	+	+			0.03	
4	+	+			0.30	
5	+	+			3.00	
6	+	+				0.03
7	+	+				0.30
8	+	+				3.00
9	+		+			
10	+		+		0.03	
11	+		+		0.30	
12	+		+		3.00	
13	+		+			0.03
14	+		+			0.30
15	+		+			3.00
16	+			+		
17	+			+	0.03	
18	+			+	0.30	
19	+			+	3.00	
20	+			+		0.03
21	+			+		0.30
22	+			+		3.00

**Inhibition Assays:** U937 cells were stimulated with PMA and incubated in 8-well plates as described previously. Aliquots of porcine MBL and human MBL were prepared and the concentrations of each were adjusted to 3 µg/ml. Anti-MBL antibody was diluted to 1 µg/ml and two-fold dilutions were prepared, beginning with the 1 µg/ml concentration. The serial dilutions were added to porcine MBL or human MBL in separate tubes and incubated for 1 hour at 4°C. 500 µl of either *Salmonella* wt ( $10^7$  CFU), the *ompD* mutant ( $10^7$  CFU), or the *ompC* mutant ( $10^7$  CFU), were added separately to 500 µl of each of the human MBL/anti-MBL dilutions, and incubated for 1 hour at 4°C. This step was repeated, but this time the bacteria were added to the porcine MBL/anti-MBL dilutions. Each assay was performed in duplicate and repeated three times. The assay involving the *ompC* mutant was performed in duplicate only. Controls were also included (see table 2.3). Bacterial concentrations were confirmed by serial plating on LB agar.

The 8-well slides containing the U937 cells were washed with HBSS two times and the two bacterial strains, previously incubated with either human MBL or porcine MBL at varying ratios, were added separately to the wells and incubated for two hours at 37°C, 5% CO<sub>2</sub>. The wells were washed 3-4 times with HBSS. Slides were fixed and stained and adherent cells were measured by light microscopy.

**Table 2.3. Contents of the various wells in the inhibition assays.**

The gray shaded areas were the controls.

Well #	U937	wt 14028	OmpD mutant	Porcine MBL	Human MBL	Anti-MBL (ratio 1:x)
1	+					
2	+	+				
3	+	+		+		
4	+	+		+		1,000
5	+	+		+		2,000
6	+	+		+		4,000
7	+	+		+		8,000
8	+	+		+		16,000
9	+	+			+	
10	+	+			+	1,000
11	+	+			+	2,000
12	+	+			+	4,000
13	+	+			+	8,000
14	+	+			+	16,000
15	+		+			
16	+		+	+		
17	+		+	+		1,000
18	+		+	+		2,000
19	+		+	+		4,000
20	+		+	+		8,000
21	+		+	+		16,000
22	+		+		+	
23	+		+		+	1,000
24	+		+		+	2,000
25	+		+		+	4,000
26	+		+		+	8,000
27	+		+		+	16,000

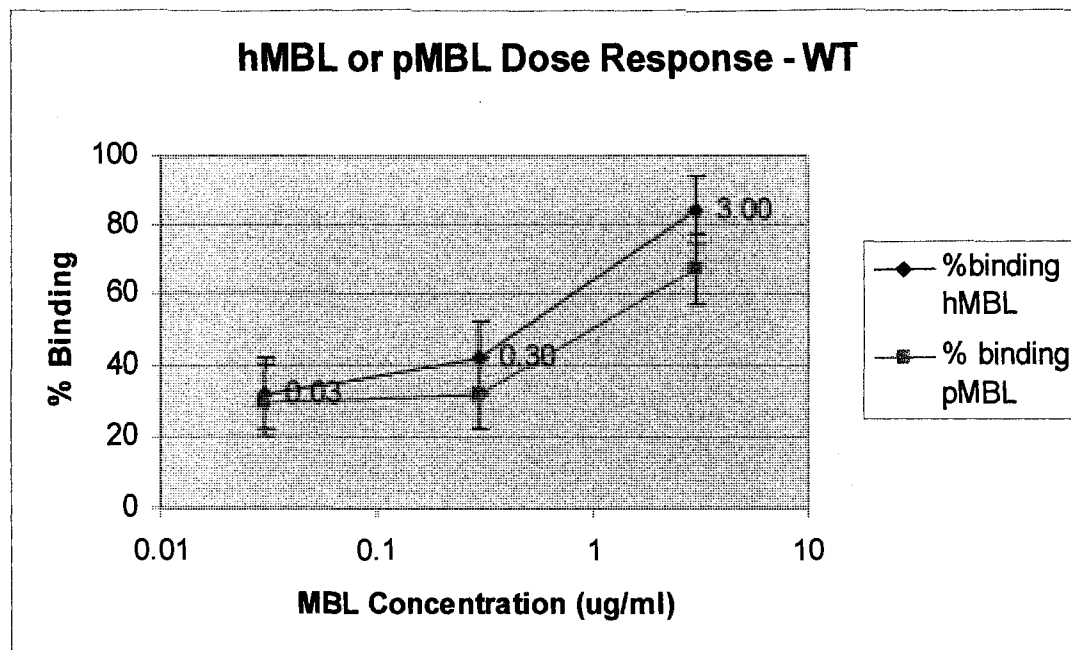
## RESULTS

**Binding activity of M $\phi$  to *Salmonella* in the presence of MBL.** In this study, a series of binding assays was performed to determine the effect of porcine MBL on the adherence of macrophages to *Salmonella* Typhimurium and two mutant strains. The results were then compared to those obtained when the bacteria were pre-incubated with human MBL. There were clear differences in binding activity between the three strains. Binding activity is illustrated in Fig. 2.1 – 2.7.

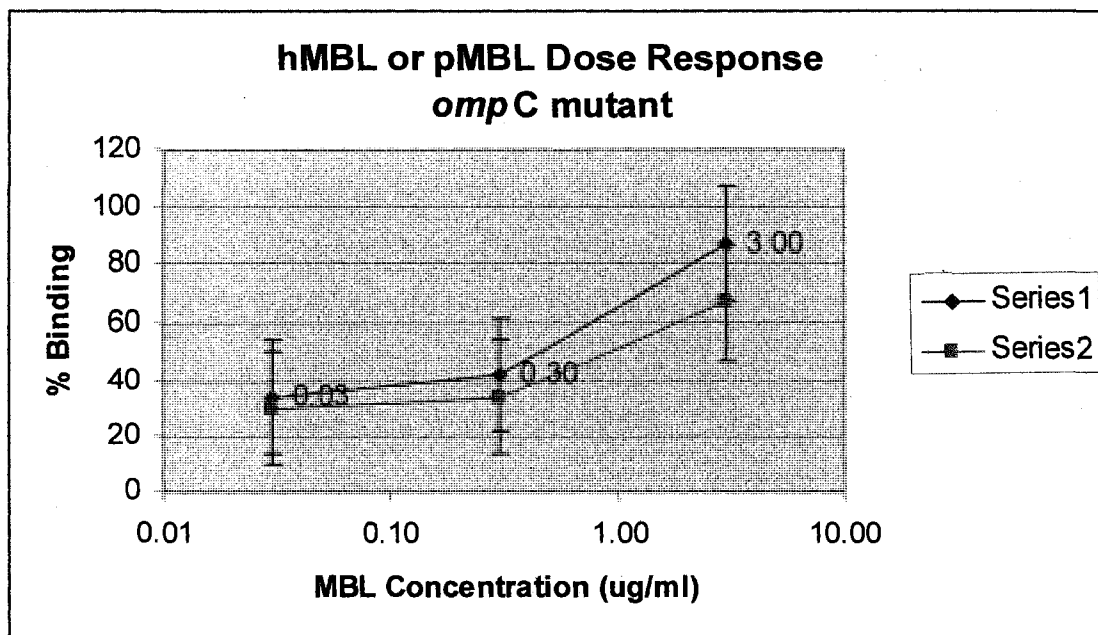
Studies involving ten-fold increases in hMBL or pMBL concentrations showed increased binding activity in a dose-responsive manner in both the wt and *ompC* mutant strains (Fig. 2.1 and Fig. 2.2). When the wt strain was preopsonized with 0.03  $\mu\text{g/ml}$  hMBL it resulted in 32% adherence; ten-fold increases in MBL concentrations resulted in 42% and 84% adherence, respectively. Preopsonization with 0.03  $\mu\text{g/ml}$  pMBL resulted in 30% adherence; ten-fold increases in MBL concentrations resulted in 32% and 67% adherence, respectively. Results were similar when using the *ompC*-mutant strain. When the *ompC* mutant was preopsonized with 0.03  $\mu\text{g/ml}$  hMBL it resulted in 34% adherence; ten-fold increases in MBL concentrations resulted in 42% and 87% adherence, respectively.

Preopsonization with 0.03 µg/ml pMBL resulted in 30% adherence; ten-fold increases in MBL concentrations resulted in 34% and 67% adherence, respectively. There was very little change in binding activity with the *ompD* mutant preopsonized with either pMBL or hMBL at any concentration of MBL. The *ompD* mutant resulted in 8% adherence with MBL concentrations up to 3.0 µg/ml.



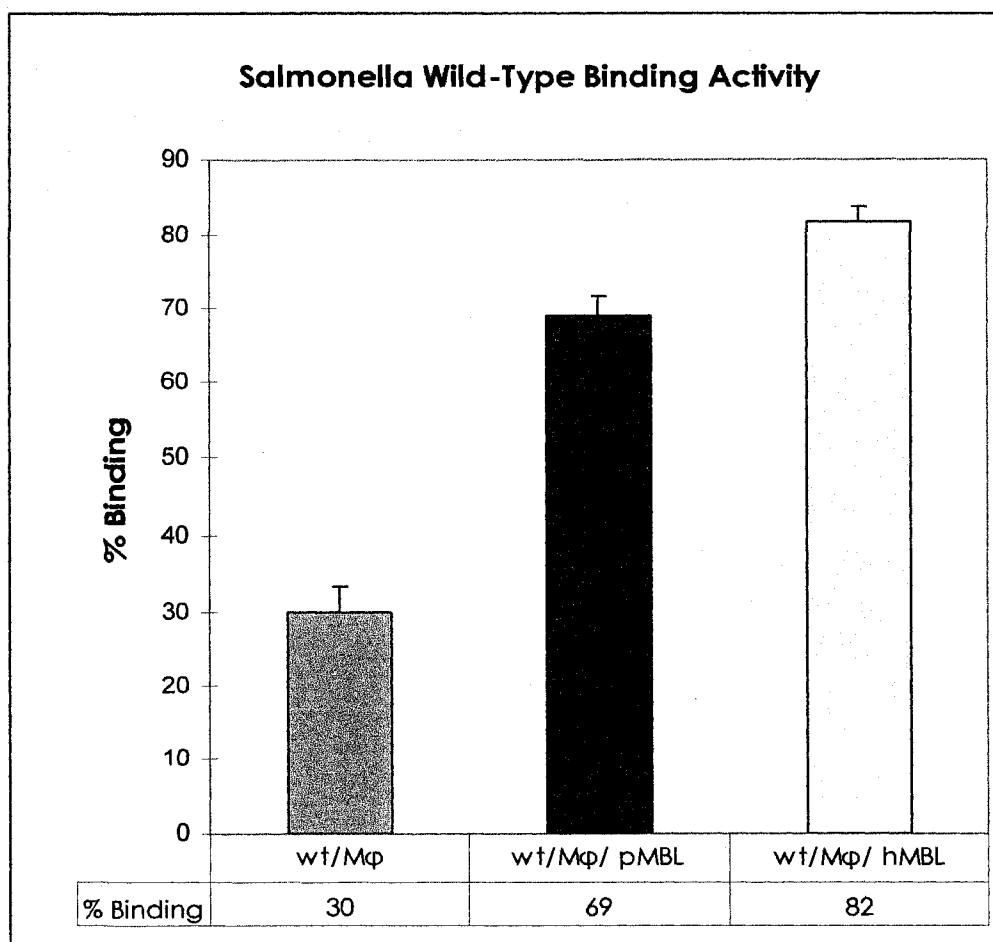


**Fig. 2.1. Wt binding activity in response to increased concentrations of MBL.** Bacteria ( $5 \times 10^7$  CFU/ml) were incubated with increasing concentrations of MBL: 0.03  $\mu$ g/ml; 0.3  $\mu$ g/ml; 3  $\mu$ g/ml for 1 h at 37°C. With the wt strain, there was increased binding activity in a dose-responsive fashion as the concentration of human or porcine MBL increased. There was not a significant increase in binding between 0.03-0.30  $\mu$ g/ml; however there was a sharp increase in adherence at 3.0  $\mu$ g/ml. Preopsonization with 0.03  $\mu$ g/ml human MBL resulted in 32% adherence; 10-fold increases in MBL concentrations resulted in 42% and 84% adherence, respectively. Preopsonization with 0.03  $\mu$ g/ml porcine MBL resulted in 30% adherence; 10-fold increases in MBL concentrations resulted in 32% and 67% adherence, respectively. Each data point represents the mean  $\pm$  SD of two separate experiments performed in duplicate.

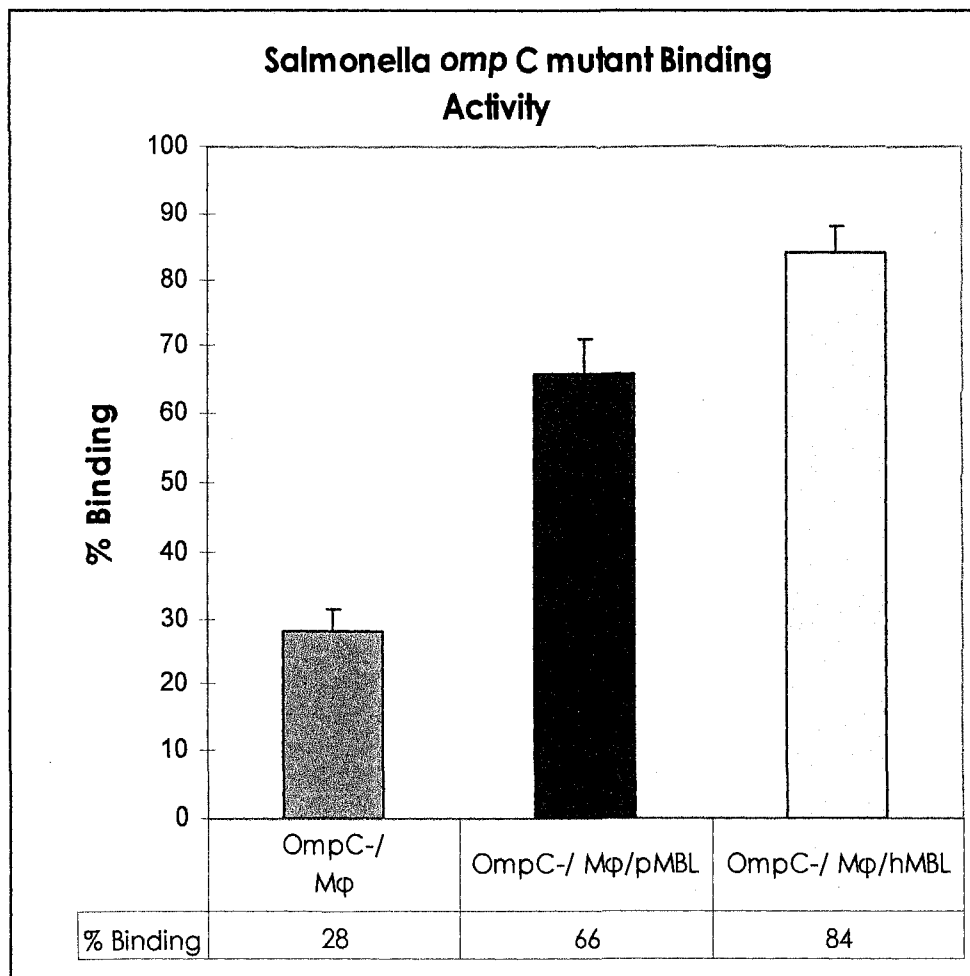


**Fig. 2.2. OmpC mutant binding activity in response to increased concentrations of MBL.** Bacteria ( $5 \times 10^7$  CFU/ml) were incubated with increasing concentrations of MBL: 0.03  $\mu\text{g/ml}$ ; 0.30  $\mu\text{g/ml}$ ; 3.0  $\mu\text{g/ml}$  for 1 h at 37°C. With the *ompC* mutant strain, there was increased binding activity in a dose-responsive fashion as the concentration of human or porcine MBL increased. There was not a significant increase in binding between 0.03-0.30  $\mu\text{g/ml}$ ; however there was a sharp increase in adherence at 3.0  $\mu\text{g/ml}$ . Preopsonization with 0.03  $\mu\text{g/ml}$  human MBL resulted in 34% adherence; 10-fold increases in MBL concentrations resulted in 42% and 87% adherence, respectively. Preopsonization with 0.03  $\mu\text{g/ml}$  porcine MBL resulted in 30% adherence; 10-fold increases in MBL concentrations resulted in 34% and 68% adherence, respectively. Each data point represents the mean  $\pm$  SD of two separate experiments performed in duplicate.

There was high binding activity of U937 cells to both the wt and *ompC* mutant strains when organisms were preopsonized with 3.0 µg/ml of hMBL or pMBL. When either bacterial strain was incubated with macrophages only (no MBL present) the binding activity averaged approximately 32%. There was a dramatic increase in binding when the wt strain was incubated with either human or porcine MBL, with 82% and 69% adherence respectively (fig. 2.3). There were similar results when the *ompC* mutant was incubated with either human or porcine MBL (fig. 2.4.) Binding activity more than doubled, with 66% adherence in the presence of pMBL and 84% adherence in the presence of hMBL. These results contrasted sharply when compared to the *OmpD*-deficient strain. There was minimal binding activity with the *OmpD* mutant strain in the presence of either human or porcine MBL, with only 8% adherence when incubated with either MBL species. There was no significant difference when comparing these results to the control, (no MBL) which exhibited 6% adherence.

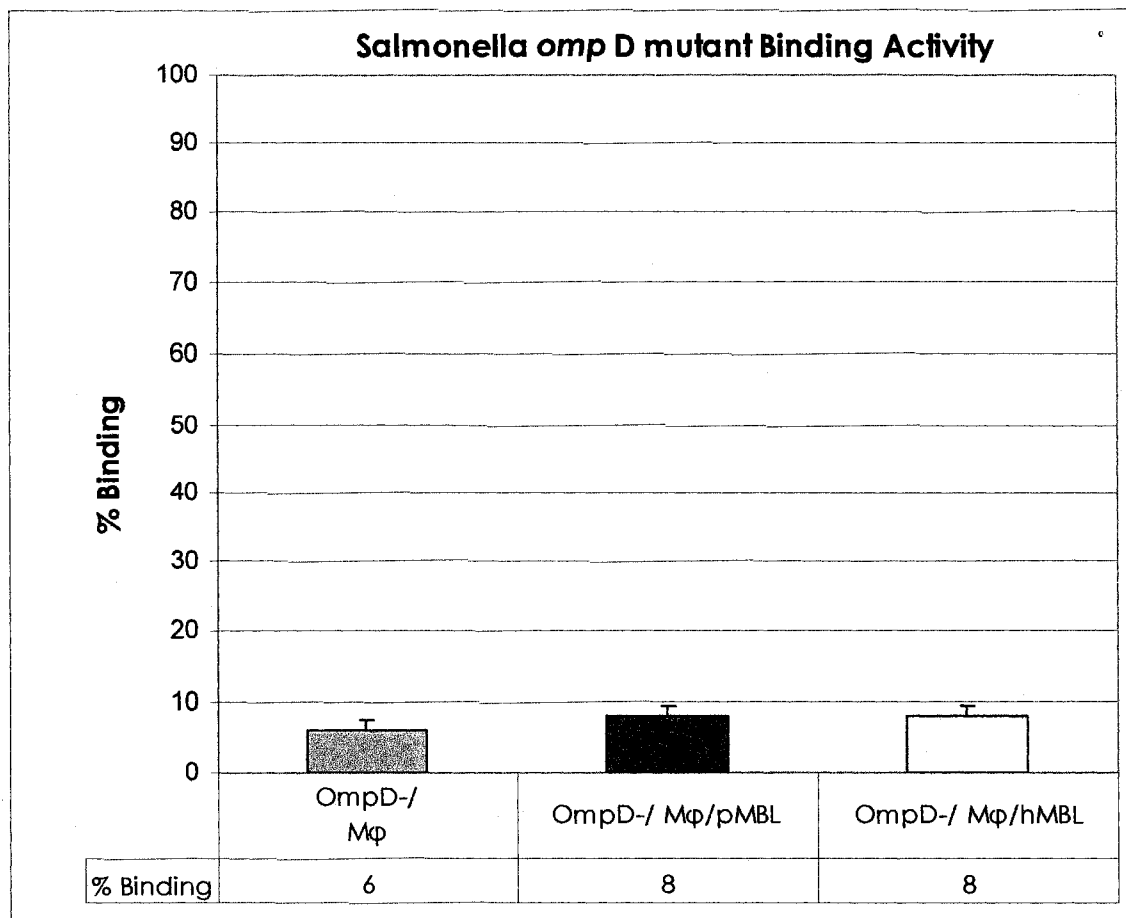


**Fig. 2.3. Binding to U937 cells by Salmonella wt incubated with 3.0 µg/ml of either human MBL or porcine MBL.** There was increased bacterial binding to U937 cells in the presence of 3.0 µg/ml of either pMBL or hMBL, with 69% adherence in the presence of pMBL and 82% adherence in the presence of hMBL. The control, without added MBL, exhibited 30% adherence.



**Fig. 2.4. Binding to U937 cells by the *Salmonella ompC* mutant strain**

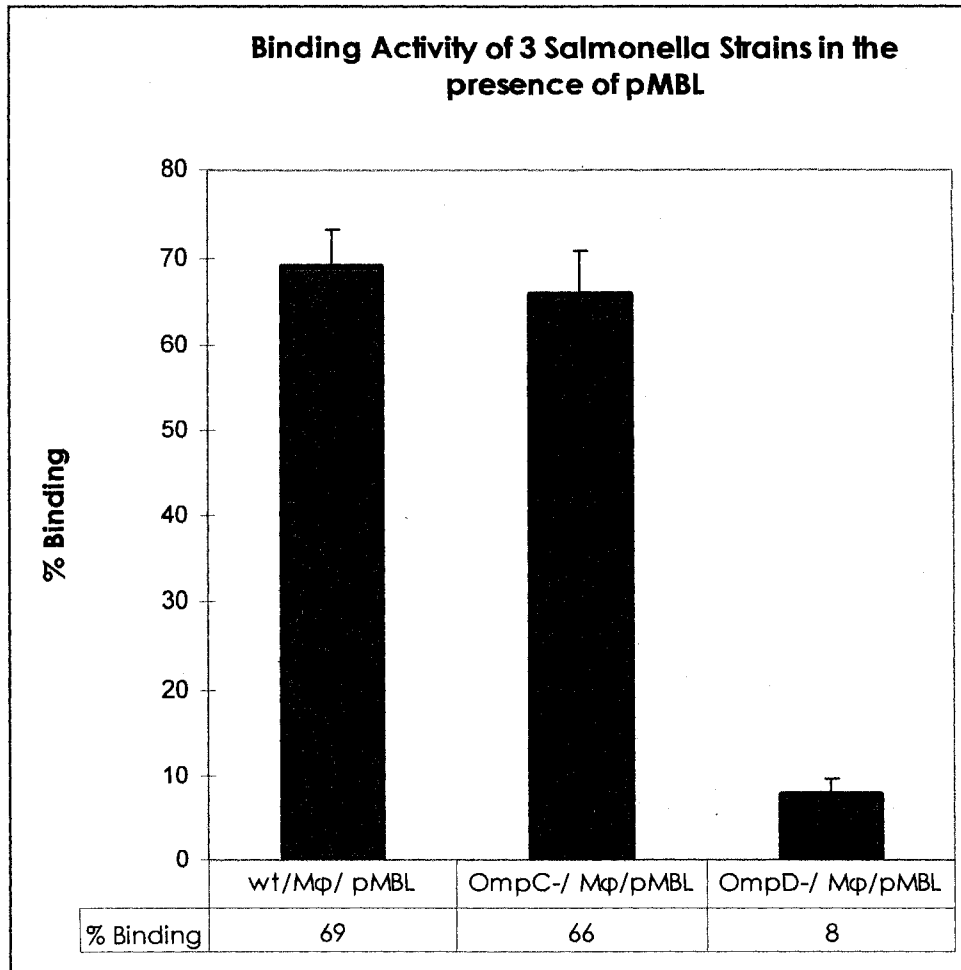
**incubated with 3.0 µg/ml of either human MBL or porcine MBL.** There was increased bacterial binding to U937 cells in the presence of 3.0 µg/ml of either pMBL or hMBL. There was 66% adherence in the presence of pMBL and 84% adherence in the presence of hMBL. The control, without added MBL, exhibited 28% adherence.



**Fig. 2.5. Binding to U937 cells by the Salmonella ompD mutant strain**

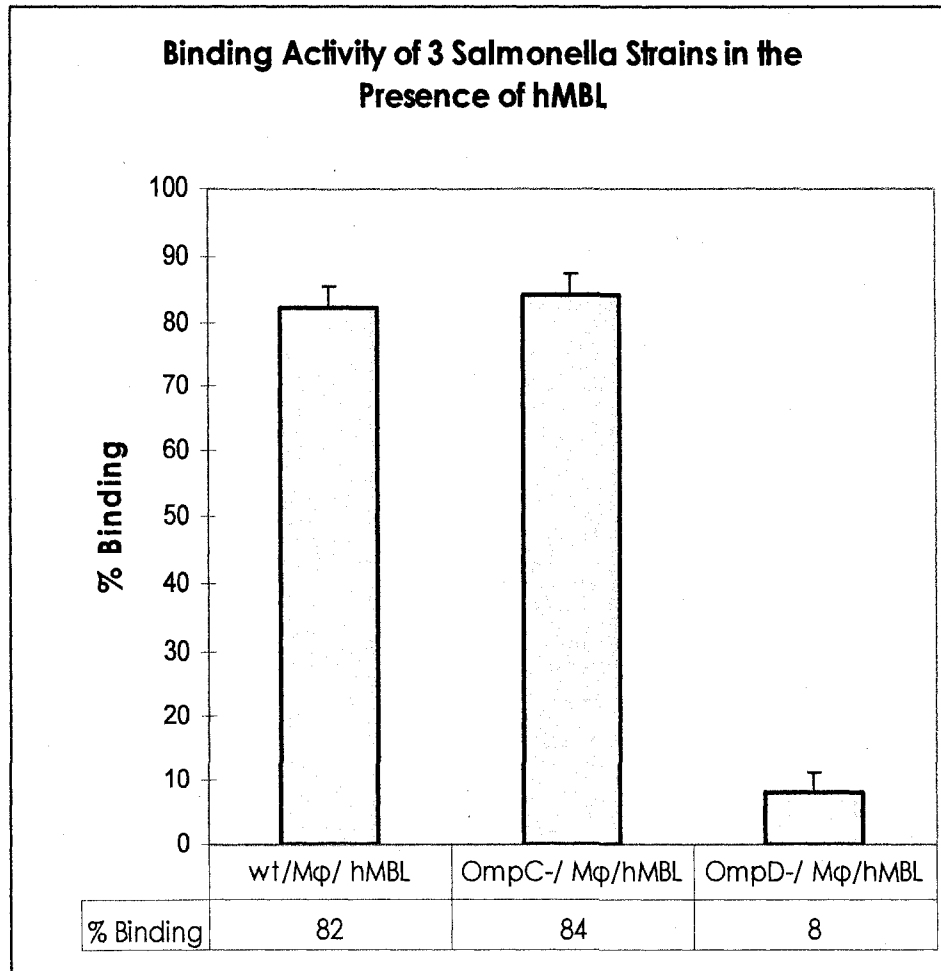
incubated with 3.0 µg/ml of either human MBL or porcine MBL. There was no statistical difference in bacterial binding to U937 cells when the ompD-deficient strain was incubated in the presence of 3.0 µg/ml of either pMBL or hMBL or when incubated without MBL. When the ompD mutant was incubated without MBL there was 6% adherence. There was 8% adherence in the presence of pMBL or hMBL.

The following two bar graphs (figs. 2.6 and 2.7) depict the same data that were presented in the previous three graphs, but emphasizing the bacterial strains as the variable, rather than the presence or absence of MBL.



**Fig. 2.6. Salmonella wild-type, *ompC* mutant and *ompD* mutant strains incubated with porcine MBL.** There was increased binding activity with the wt and *ompC* mutant strains in the presence of pMBL, with 69% and 66% adherence respectively. There was 8% adherence with the *ompD*-deficient strain in the presence of pMBL.





**Fig. 2.7. Salmonella wild-type, *ompC* mutant and *ompD* mutant strains incubated with human MBL.** There was increased binding activity with the wt and *ompC* mutant strains in the presence of hMBL, with 82% and 84% adherence respectively. There was 8% adherence with the *ompD*-deficient strain when incubated with hMBL.

### **The effects of inhibition of MBL on *Salmonellae* adherence to U937**

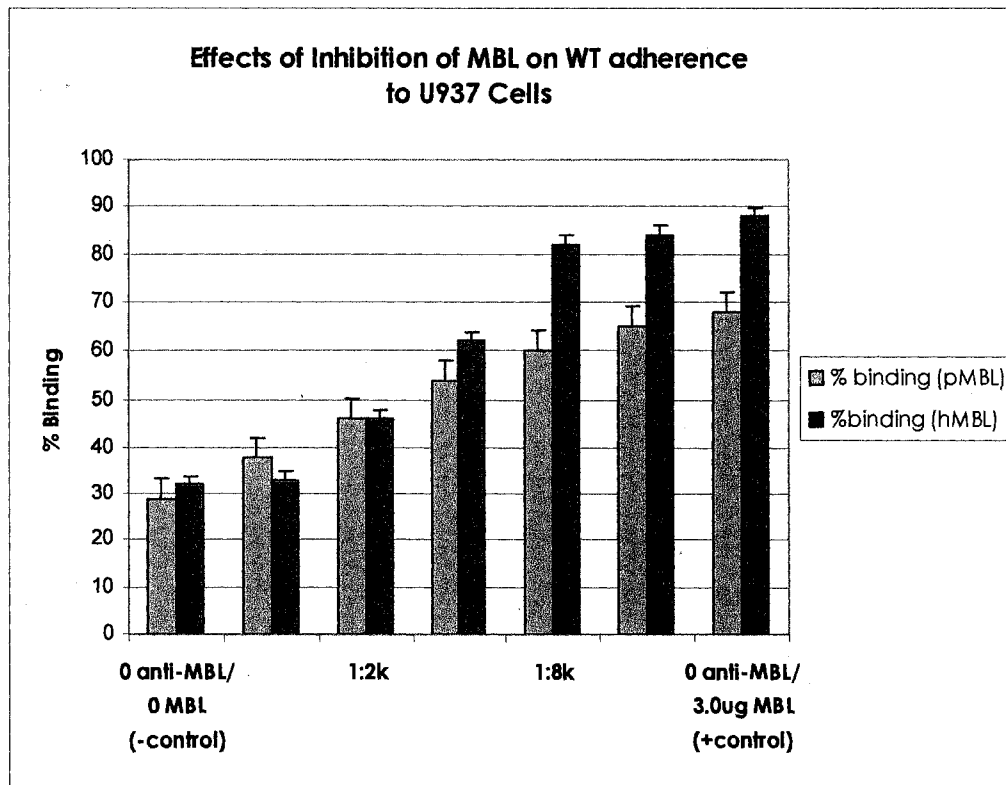
**Cells.** We examined the inhibitory potential of anti-porcine MBL monoclonal antibody toward both human or porcine MBL and its effects on bacterial adherence to macrophages. Since very little binding activity was observed when the *ompD* mutant was incubated with the U937 cells, with or without either pMBL or hMBL, it was expected that there would be little to no change in the *ompD* mutant binding activity when the anti-MBL antibody was added.

PMA-stimulated U937 cells were incubated with *Salmonella* wt, *ompC* mutant or *ompD* mutant strains. Two-fold dilutions of anti-MBL antibody were incubated with either porcine or human MBL. Finally, either the wt, *ompC*, or *ompD* mutant strains were incubated separately with each of the hMBL/anti-MBL or pMBL/anti-MBL dilutions. Increased binding activity was observed in the wt strain as the anti-MBL concentrations decreased, until it reached a plateau between the 1:8k and 1:16k dilution in the presence of either pMBL or hMBL. The two negative controls (-pMBL/-antibody; -hMBL/-antibody) showed 29% and 32% adherence respectively. The two positive controls (+pMBL/-antibody; +hMBL/-antibody), showed 64% and 87% binding, respectively. Figure 2.8 shows the % binding activity that occurred in the presence of either pMBL or

hMBL at the different anti-MBL dilutions.

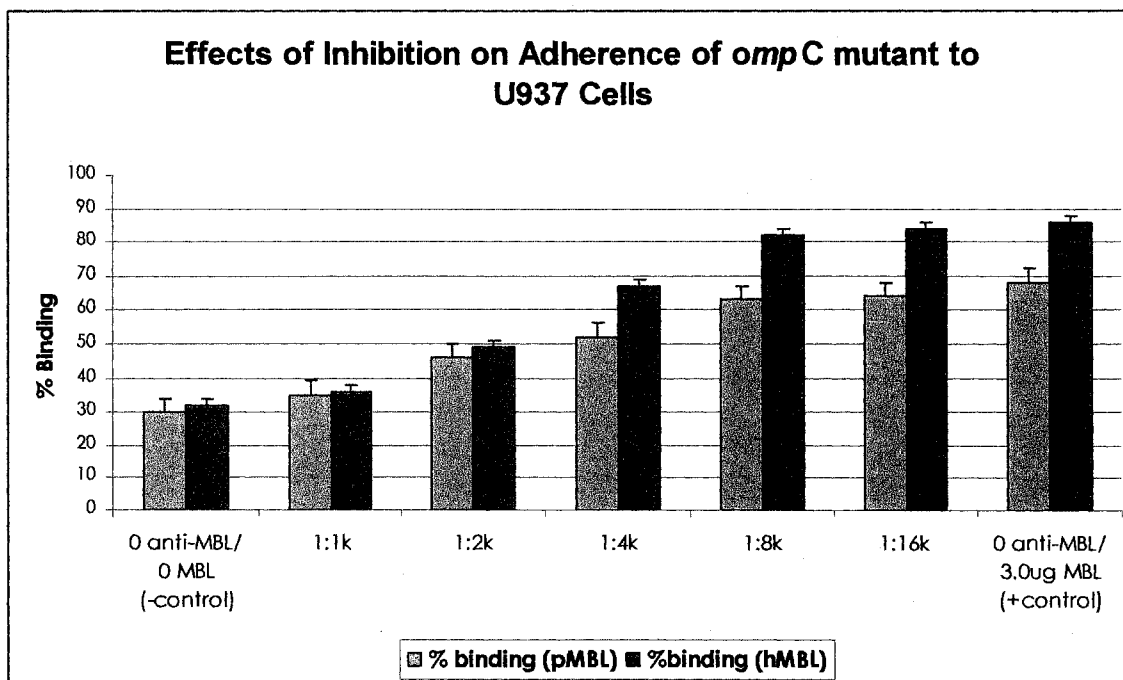
A similar trend was observed in the *ompC* mutant strain, i.e., as the anti-MBL concentrations decreased adherence increased. The two negative controls (-pMBL/-antibody; -hMBL/-antibody) showed 30% and 32% adherence respectively. The two positive controls (+pMBL/-antibody; +hMBL/-antibody), showed 68% and 86% binding, respectively.

The *ompD* mutant strain showed no difference in adherence to U937 cells in the presence of decreasing concentrations of anti-MBL. The binding activity was similar to the results in the binding assays performed without MBL, i.e., approximately 6%-8% . The two negative controls (-pMBL/-antibody; -hMBL/-antibody) showed 8% and 6% adherence respectively. The two positive controls (+pMBL/-antibody; +hMBL/-antibody), both showed 8% adherence (fig. 2.10).

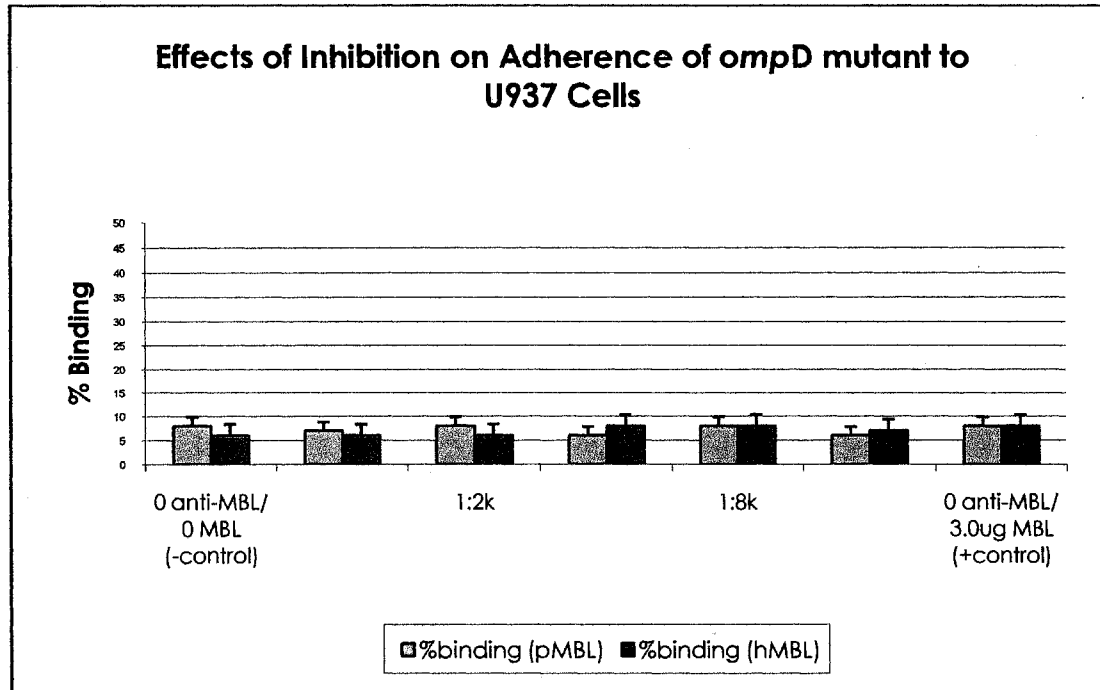


**Fig. 2.8. The effects of inhibition of MBL on wt adherence to U937 cells.**

Increased binding activity was observed in the wt strain as the anti-MBL concentrations decreased, until it reached a plateau between the 1:8k and 1:16k dilution in the presence of either pMBL or hMBL. The two negative controls (-pMBL/-antibody; -hMBL/-antibody) showed 29% and 32% adherence respectively. The two positive controls (+pMBL/-antibody; +hMBL/-antibody), showed 64% and 87% binding, respectively.



**Fig. 2.9. The effects of inhibition of MBL on *ompC*- deficient *Salmonella* adherence to U937 cells.** Increased binding activity was observed in the *ompC*- deficient strain as the anti-MBL concentrations decreased, until it reached a plateau between the 1:8k and 1:16k dilution in the presence of either pMBL or hMBL. The two negative controls (-pMBL/-antibody; -hMBL/-antibody) showed 30% and 32% adherence respectively. The two positive controls (+pMBL/-antibody; +hMBL/-antibody), showed 68% and 86% binding, respectively.



**Fig. 2.10. The effects of inhibition of MBL on *ompD*- deficient *Salmonella* adherence to U937 cells.** The *ompD* mutant strain showed no difference in binding activity as the anti-MBL concentrations decreased in the presence of either pMBL or hMBL. The two negative controls (-pMBL/-antibody; -hMBL/-antibody) showed 8% and 6% adherence respectively. The two positive controls (+pMBL/-antibody; +hMBL/-antibody), both showed 8% adherence.

## DISCUSSION

The role of OmpD in pathogenesis has been widely questioned for many years. Some studies have shown that certain *ompD*- deficient *Salmonella* strains were less virulent than the wild-type parent strains, while others have reported that there is no difference in virulence (11,12,29,30). Previously in our lab it was shown that OmpD was involved in adherence of *Salmonella* Typhimurium 14028 to macrophages (29,30). Our present data also show that there was a difference between the abilities of two different *Salmonella omp*- deficient strains and their parent strain to bind to U937 cells under anaerobic conditions. In addition, there was virtually no difference in binding activity in the presence of either porcine or human MBL with the *ompD* mutant strain.

There are many possible explanations for these results. During the infection process *Salmonella* is exposed to many extreme environmental changes, including variations in O<sub>2</sub> levels and pH, and consequently have developed various strategies to survive these changes (7,12). Under aerobic conditions OmpD is the primary porin present in *Salmonella* wild-type cells, in approximately  $1 \times 10^5$  porin molecules per cell (47). Collectively, OmpD, OmpC, and OmpF are present in approximately

$2 \times 10^5$  porin molecules per cell (47). Conversely, under anaerobic conditions the amount of OmpD porin molecules almost doubles when compared to the expression of other outer membrane proteins, which either decrease or remain relatively stable in quantity (47). It is possible that when the OmpD porin is eliminated the cell surface has altered in such a way that it no longer allows binding of the OmpD to the U937 cell line. Since OmpD is the major porin protein present in *Salmonella* wild-type cells, its deletion would drastically change the overall protein framework of the outer membrane. In addition, LPS and porins are so tightly associated that it is extremely difficult to separate the two during purification processes (26). Thus, the deletion of one may significantly affect the other. For example, Koplow and Goldfine (44) found that levels of major outer membrane proteins were drastically decreased in *S. Typhimurium* mutants expressing very defective LPS. This external structural change caused the mutants to become very sensitive to certain hydrophobic substances. Conversely, when an outer membrane porin is eliminated it is possible that the LPS may be defective. This could also explain why MBL had very little effect on ompD adherence. Since MBL binds to *Salmonella* by means of a pattern-recognition mode of detection (4,25,26) and the spacing and orientation of the CRD defines what ligands MBL can target (7,28), then it would follow that if the LPS has been altered



then binding may be greatly affected.

The two MBL species showed different levels of binding activity. Although the wt and *ompC* mutant strains showed increased adherence as the human or porcine MBL concentrations increased, pMBL had less effect than hMBL. Human and porcine MBL share approximately 67% homology (20). Although this percentage is relatively high there is also a 33% difference. Human MBL may possess one or more epitopes that recognize certain surface molecules on *Salmonella* that pMBL does not. The presence of such an epitope may assist in the binding process and therefore result in higher binding activity. There was a difference between the two MBL species in the amount of binding observed; however, as mentioned previously there were similarities in the overall patterns in adherence, ie., binding increased as the MBL concentrations increased. Based on these results, it appears that both human MBL and porcine MBL have analogous effects on *Salmonella* adherence to U937 cells.

The porcine MBL was purified by using a multi-step chromatography process. The second phase involved a protein A column. This method was a novel approach and based on SDS-PAGE and ELISA results,

appears to have successfully separated the IgG from the MBL. However, the purified porcine MBL sample may have contained a small amount of residual protein. To further verify the purity of the sample, we would suggest that additional tests be performed in the future, such as mass spectrometry and/or genetic testing. The results of these tests may warrant the addition of a third phase in the purification process, such as ion-exchange chromatography or chromatofocusing.

We conclude that both human and porcine MBL are opsonins that are able to increase macrophage binding to *Salmonella* in a dose-dependent fashion. In addition, our results suggest that the outer membrane protein porin D of *Salmonella* wild-type strain 14028, is a ligand for adherence to the macrophage cell line U937.

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